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Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats

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***In vitro* expansion of central nervous system (CNS) precursors might overcome the limited availability of dopaminergic neurons in transplantation for Parkinson's disease, but generating dopaminergic neurons from *in vitro* dividing precursors has proven difficult. Here a three-dimensional cell differentiation system was used to convert precursor cells derived from E12 rat ventral mesencephalon into dopaminergic neurons. We demonstrate that CNS precursor cell populations expanded *in vitro* can efficiently differentiate into dopaminergic neurons, survive intra-striatal transplantation and induce functional recovery in hemiparkinsonian rats. The numerical expansion of primary CNS precursor cells is a new approach that could improve both the ethical and the technical outlook for the use of human fetal tissue in clinical transplantation.**

Parkinson's disease is a neurodegenerative disorder affecting an estimated one million patients in the United States alone. Several strategies are being pursued to develop new therapies for Parkinsonian patients. These techniques range from the use of dopaminotrophic factors¹ and viral vectors² to the transplantation of primary xenogeneic tissue³. Fetal nigral transplantation is a clinically promising experimental treatment in late stage Parkinson's disease (PD). More than two hundred patients have received transplants worldwide⁴. Clinical improvement has been confirmed by functional studies using positron emission tomography of striatal fluorodopa uptake after transplantation^{4,5} and was correlated to good graft survival and innervation of the host striatum in postmortem studies of transplanted patients⁶. Two controlled clinical studies, sponsored by the US National Institutes of Health, are ongoing in the United States using fetal mesencephalic tissue in a larger number of patients and a large multicenter study is being planned in Europe. In spite of these promising findings, neural transplantation remains a controversial procedure. Successful numerical expansion of primary CNS precursors could alleviate some of the ethical and technical difficulties involved in the use of human fetal tissue.

There has been considerable recent progress in characterizing stem cells from the central and peripheral nervous systems *in vitro* and *in vivo*⁷⁻¹¹. However, controlled conversion of neuroepithelial precursors into dopaminergic neurons has not been reported. Here we demonstrate that precursor cells obtained from the rat embryonic day 12 (E12) ventral mesencephalon can be expanded *in vitro* and differentiated into dopaminergic neurons. Upon transplantation, differentiated precursors alleviate behavioral deficits in a rat model of Parkinson's disease.

Results

CELL EXPANSION

An overview of the experimental procedure is shown in Fig. 1. Cells were obtained from the ventral mesencephalon of E12

rat embryos, mechanically dissociated, plated on polyornithine/fibronectin-coated culture dishes and grown in serum-free medium supplemented with basic fibroblast growth factor (bFGF). No cells immunoreactive for the rate-limiting enzyme in the synthesis of dopamine, tyrosine hydroxylase (TH), could be detected in the tissue at the time of dissection. In response to bFGF, cells proliferated and formed cell clusters that grew to macroscopic size after one week *in vitro* (Fig. 2a). Within seven days *in vitro*, the total cell number increased from 800,000 to $7,417,000 \pm 1,066,000$ cells, ($n = 12$, Fig. 2b). Cultures grown without bFGF had a substantial net cell loss. Cells not responding to the mitogenic activity of bFGF, as assessed by time-lapse photomicrography, were prone to cell death (Fig. 2d and e), determined by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end label (TUNEL) method. Cell death within proliferating clusters was relatively rare (Fig. 2f). Immunohistochemistry showed that bFGF receptors were present in most cells ($88 \pm 4\%$) throughout expansion. After six to eight days of bFGF treatment, all surviving cells were growing in clusters and were immunoreactive for the nestin intermediate filament protein, a marker for immature neuroepithelial precursors^{12,13} (Fig. 2c).

CELL DIFFERENTIATION

Removal of the mitogen initiates differentiation of nestin-immunoreactive precursor cells *in vitro*⁷. Upon removal of bFGF from the culture medium, extensive arborizations developed, interconnecting large clusters of cell bodies. Cell bodies and fiber bundles were strongly immunoreactive for β -tubulin type III. After seven days of differentiation, neuronal morphologies were more mature, and $18.4 \pm 5.1\%$ of the total cell population was immunoreactive for TH ($n = 19$, Fig. 3a). The TH-immunoreactive cells were also immunoreactive for dopamine (Fig. 3a, inset), dopamine transporter and β -tubulin type III (data not

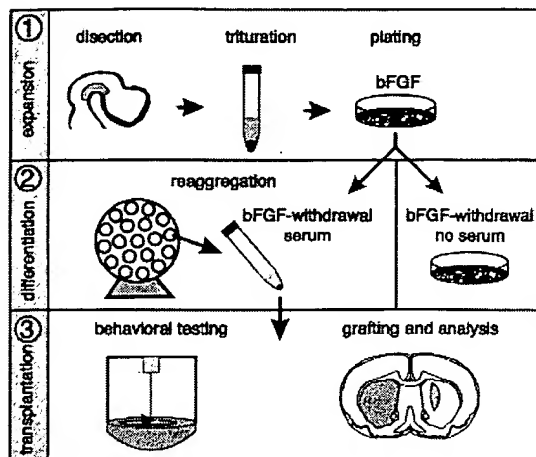


Fig. 1. Schematic illustration of the experimental procedures. **Phase 1** (cell expansion), Following tissue dissection and trituration to single-cell density, precursor cells were expanded for one week in serum-free medium supplemented with bFGF and grown on precoated culture dishes. **Phase 2** (cell differentiation), Conversion of dividing precursors into dopaminergic neurons was induced by withdrawal of the mitogen. Two cell differentiation systems have been developed. Cultures growing under serum-free conditions and attached to culture plate are differentiated by bFGF withdrawal only (right box). Cultures raised for transplantation purposes were differentiated as free-floating aggregates in a medium containing 10% fetal bovine serum (FBS) and maintained in a roller drum system (left box). **Phase 3** (cell transplantation), Differentiated aggregates were transplanted into the ipsilateral striatum of 6-hydroxydopamine-lesioned rats. Graft function was assessed as changes in amphetamine-induced rotation behavior.

shown). In non-expanded cultures, grown without bFGF (Fig. 2b), $5.6 \pm 1.3\%$ of cells were TH immunoreactive.

Thus expansion and differentiation of precursors increased by threefold the percentage of neurons that were dopaminergic (18.4%), compared to non-expanded cultures (5.6%). This number, multiplied with a tenfold increase in total cell number during the expansion phase, leads to an estimated 30-fold increase in the *in vitro* yield of nigral dopaminergic neurons. One E12 embryo yielded 100,000–120,000 nigral cells at the day of dissection. After these cells were expanded tenfold, 18.4% of them stained positive for TH after cell differentiation, yielding 180,000–220,000 TH-immunoreactive cells. When compared to the total number of dopaminergic neurons present in the adult rat

(26,000–30,000)^{14,15}, the expansion procedure leads to an estimated sevenfold increase in TH-immunoreactive cell number.

Transplantation of cells that are differentiated on culture dishes entails mechanical or enzymatic dissociation, with subsequent disruption of axodendritic trees and cell loss. Therefore we developed a reaggregation system that allows conversion of previously expanded precursors into dopaminergic neurons in free-floating spheres (Fig. 3b). These reagggregates can be directly loaded into a stereotactic needle and transplanted as a unit. Under differentiation conditions, reaggregate size increased over six to eight days, resulting in a spherical culture of 0.6–1.2 mm in diameter. No substantial cell proliferation occurred during the differentiation phase, as shown by immunohistochemistry for

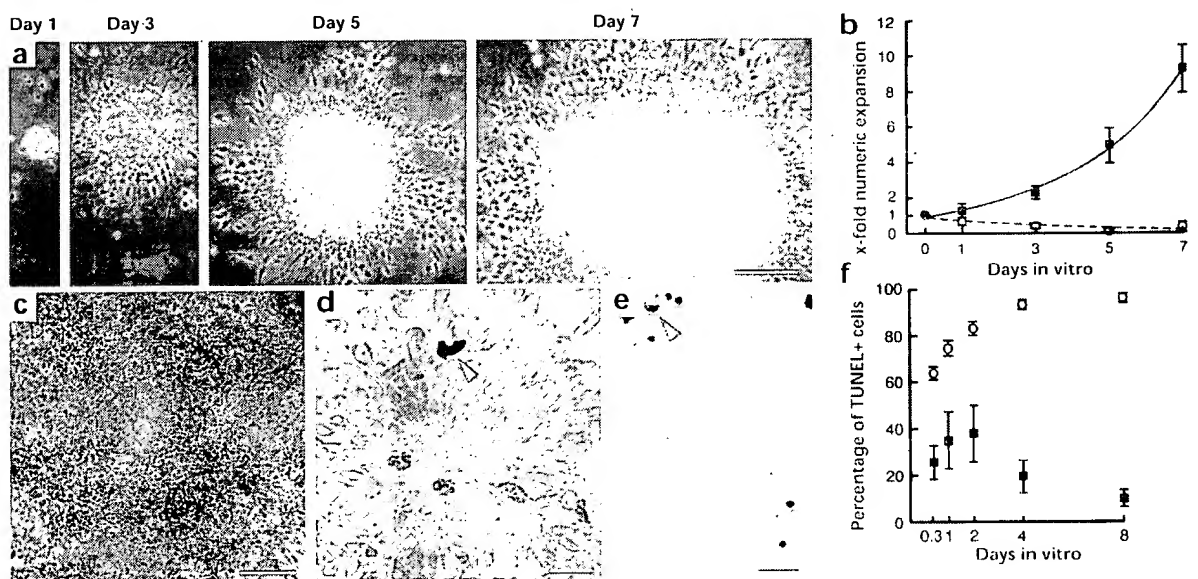
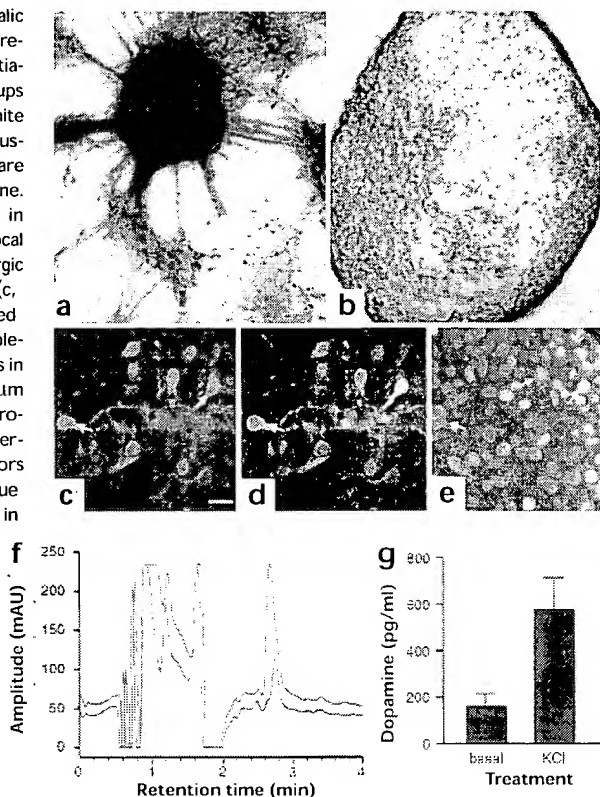


Fig. 2. Expansion of mesencephalic precursors *in vitro*. (a) Time-lapse, phase-contrast photomicrography during bFGF expansion. (b) Comparison of growth rate between bFGF treated and control cultures. ■ bFGF, ○ control. (c) Expanded cells are immunoreactive for nestin (seven days of bFGF treatment). (d, e) TUNEL-positive nuclei (arrowheads) in clusters of dividing cells (d) and in single cells (e) after two days of bFGF treatment. (f) Single cells demonstrate a gradual increase in TUNEL labeling, reaching $96 \pm 2\%$ TUNEL-positive cells at the end of expansion phase. Cell clusters had only a small percentage of TUNEL-positive cells at the end of the expansion phase. ■ cells in clusters, ○ single cells. Scale bars, 100 μ m (a, c); 10 μ m (d, e).

Fig. 3. Differentiated mesencephalic precursors *in vitro*. **(a)** TH-immunoreactive neurons seven days after initiation of cell differentiation. Small groups of TH-immunoreactive cells (white arrow) migrated out of the large cluster. Inset, differentiated precursors are also immunoreactive for dopamine. **(b)** TH-immunoreactive neurons in free-floating aggregates. **(c–e)** Confocal images of differentiated dopaminergic neurons double labeled for TH (c, green label, and d) and BrdU (c, red label, and e). Three typical double-labeled cells are marked with arrows in (c–e). Scale bars, 100 μ m (a, b); 10 μ m (c–e). **(f)** Representative HPLC chromatogram of the conditioned supernatant of differentiated precursors exposed to HBSS for 25 minutes (blue curve) or exposed to 56 mM KCl in HBSS for the same time period (red curve). The retention time of dopamine was 2.7 minutes. **(g)** Quantification of the dopamine levels in the supernatant under basal conditions (HBSS only, blue column) or under stimulation with KCl (red column). Data are mean \pm standard error of three independent culture series.



the proliferation marker Ki-67, which confirmed that only a small fraction ($1.2 \pm 0.9\%$, $n = 10$) of the cells were mitotically active after seven days of differentiation.

Supplementation of the medium with 10% fetal bovine serum (FBS) led to a dramatic increase of TH-immunoreactive neurons in differentiated reaggregates with 6178 ± 547 TH-immunoreactive neurons per sphere, as compared to 1341 ± 341 in cultures grown without FBS ($p < 0.01$). The average volume of serum-treated reaggregates, was $0.77 \pm 0.11 \text{ mm}^3$, resulting in a mean density of $10,749 \pm 2386$ TH-immunoreactive cells per cubic mm. The percentage of TH-immunoreactive cells was $14.1 \pm 4.2\%$ in serum-treated reaggregates and $2.9 \pm 1.3\%$ in the control group. ($n = 21$ for FBS-treated group; $n = 11$ for control group). Treatment with GDNF (10 ng per ml), BDNF (10 ng per ml), NT4/5 (10 ng per ml) or SHH (2.5 μ g per ml) did not significantly increase the total number of TH-immunoreactive cells per sphere in serum-supplemented medium (data not shown). However, under serum-free conditions, GDNF did significantly improve TH-immunoreactive cell yield as compared to control cultures (GDNF 3451 ± 621 , $n = 8$; control group 1341 ± 341 , $n = 11$; $p < 0.05$). Because no growth factor could substantially exceed the effect of FBS, cultures produced for transplantation purposes were raised in 10% FBS without any additional growth factors.

Other neuronal phenotypes present in 10% FBS differentiated reaggregates were, in descending order of frequency, GABAergic (10–15%), serotonergic (3–5%) and cholinergic ($< 0.1\%$) neurons. The percentage of glial cells in differentiated reaggregates was low. In serum-treated cultures, $2.8\% \pm 1.2\%$ ($n = 9$) of the total cell population was immunoreactive for glial

fibrillary acid protein (GFAP), an astrocytic marker. The percentage of astrocytes in serum-free cultures was even lower ($1.1 \pm 0.9\%$, $n = 9$). No mature galactocerebroside-immunoreactive oligodendrocytes could be detected in differentiated reaggregates under any of the conditions tested.

Cultures were exposed to 1 μ M BrdU throughout the expansion phase and analyzed by confocal microscopy with z-axis sectioning for TH and BrdU double labeling after differentiation. There was no significant difference in BrdU labeling between TH-immunoreactive cells differentiated in reaggregate cultures and TH-immunoreactive cells differentiated attached to the culture plate. An average of $41 \pm 14\%$ ($n = 12$) of all dopaminergic cells had clearly incorporated BrdU (Fig. 3c). The percentage of BrdU labeling in the total cell population was $47 \pm 21\%$ ($n = 12$). This is a minimum estimate, as high-

er BrdU concentrations (10 μ M) led to increased labeling rates but dramatically reduced overall cell viability (data not shown). Reverse-phase high-performance liquid chromatography (HPLC) with electrochemical detection demonstrated that one milliliter of medium collected from expanded precursors after five to seven days of differentiation contained between 80 and 250 picograms of dopamine. Similar dopamine levels were detected in the supernatant of differentiated cultures maintained in Hank's balanced buffer solution (HBSS) for 25 minutes (162 ± 54 pg dopamine per ml HBSS, $n = 3$) (Fig. 3f and g). Evoked release by exposing cultures to 56 mM KCl in HBSS for 25 minutes led to a three- to fourfold increase in dopamine levels in the supernatant (572 ± 145 pg dopamine per ml, $n = 3$) (Fig. 3f and g). These data demonstrate that dividing precursor populations efficiently generate dopaminergic neurons.

RESULTS IN VIVO

In vivo function of reaggregate cultures was tested by transplantation into the ipsilateral striatum of adult hemiparkinsonian rats ($n = 7$). All animals had been previously lesioned by unilateral injections of 6-hydroxydopamine into the medial forebrain bundle and displayed a stable rotation response to amphetamine (average 11 ± 2 rotations per min). Gradual behavioral recovery was observed in transplanted animals (Fig. 4c). Each animal had received six to seven reaggregates, corresponding to a total number of $3\text{--}4 \times 10^5$ grafted cells. Eighty days after transplantation, rotation scores had improved substantially in five of seven animals. Rotation scores for these five animals were reduced 75% on average compared to pretransplantation scores (range 47 to

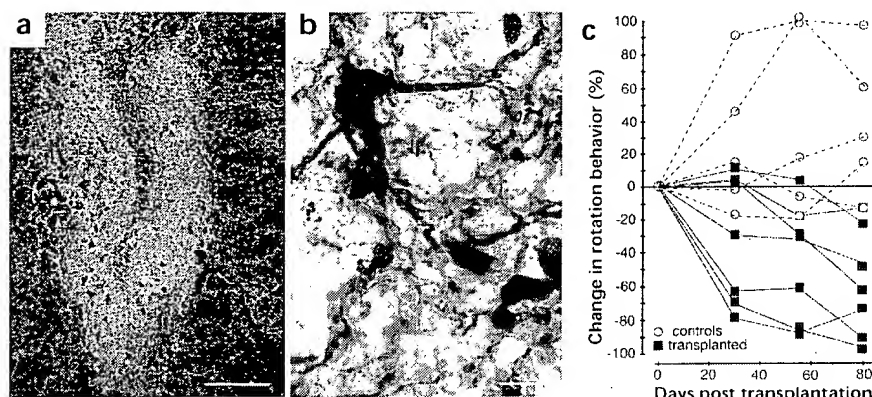


Fig. 4. *In vivo* results. (a) TH-immunoreactive graft in adult hemiparkinsonian rat 80 days after transplantation. (b) Differentiated dopaminergic neurons in graft. (c) Time course of amphetamine-induced rotation response. Data are given as mean change in rotation scores for each animal as compared to pretransplantation values (pretransplantation scores were 11 ± 2 rotations per min in both groups; $n = 7$, transplanted group; $n = 5$, control group). Significant differences between transplanted and control animals could be detected at all three time points after transplantation ($p < 0.05$). Scale bars, 200 μm (a); 10 μm (b).

97%). The remaining two animals showed only mild improvement (13% and 22% reduction). Control animals showed no behavioral improvement.

Immunohistochemical analysis of the substantia nigra, ventral tegmental area and striatum confirmed that all animals included in this study had a complete lesion. All animals had viable grafts 80 to 101 days after transplantation. The number of surviving TH-immunoreactive cells per graft was 1221 ± 431 , and the average graft volume was $0.92 \pm 0.12 \text{ mm}^3$, resulting in a TH-immunoreactive cell density of 1369 ± 389 cells per mm^3 . Graft size and TH-immunoreactive cell density in this study were similar to those found after transplanting primary fetal mesencephalic tissue¹⁶. A graft with only 478 surviving TH-immunoreactive cells and a cell density more than threefold lower than average was found in one of the two animals without significant behavioral recovery. The other animal with only mild behavioral improvement had an average number of surviving TH-immunoreactive cells, but the graft was located ectopically. Cells in all of the grafts displayed morphological features of mature dopaminergic neurons and a strong immunoreactivity for TH (Fig. 4b). Moderate fiber outgrowth from the graft into the host brain was found (Fig. 4a), comparable to results from grafted primary fetal mesencephalic cell cultures¹⁶. These results show that grafted dopaminergic neurons derived from expanded precursors are functional *in vivo* and can alleviate behavioral deficits in an animal model for Parkinson's disease.

Discussion

Cell grafting in Parkinsonian models has used primary mesencephalic cells^{4,17} or genetically modified cells that do not differentiate into neurons in the host striatum^{18–20}. Here we present the first evidence that dopaminergic neurons derived from precursors expanded *in vitro* function *in vivo*. There are three main advantages of using expanded precursors in the treatment of Parkinson's disease. First, the expansion of cell number in the proliferative phase and the enrichment in dopaminergic neurons in the differentiation step can reduce the amount of tissue need-

ed for therapeutic grafting. Second, the culture period provides an opportunity to genetically manipulate the cultured cells to optimize neuronal survival^{21,22} and fiber outgrowth¹ after grafting. Third, the controlled *in vitro* generation of dopaminergic neurons is an ideal tool to investigate the function of new genes recently implicated in the differentiation of dopaminergic neurons^{23–30}.

Although non-expanded precursors transplanted into the developing brain have shown an extraordinary plasticity in neuronal differentiation^{31–34}, *in vitro* expanded cells have been much less successful in generating neurons, especially when grafted into the adult CNS^{35,36}. We therefore differentiated precursor cells *in vitro* to obtain stable neuronal phenotypes prior to transplantation. Work with primary fetal

mesencephalic tissue suggests that behavioral recovery is directly correlated to the number of surviving dopaminergic neurons in the host striatum³⁷. In this study, we did not use a large enough number of animals to conclusively address this issue for expanded precursors. Although unlikely, non-dopaminergic effects such as the secretion of neurotrophic factors by the transplanted cells could have partly contributed to the behavioral recovery. Here, the survival rate of grafted dopaminergic neurons (3–5%) derived from expanded precursors is similar to those reported for primary nigral cells transplanted into the striatum of rodents or Parkinson's patients⁴. The successful grafting results demonstrate that long-term survival and functional integration into the adult CNS can be achieved with expanded and predifferentiated precursors. The design of similar *in vitro* systems for other neuronal phenotypes could substantially widen the use of CNS precursor cells in neurodegenerative diseases.

Neurotrophic factors have been characterized that protect dopaminergic neurons in animal models for Parkinson's disease and promote the survival and fiber outgrowth of transplanted fetal dopaminergic neurons^{1,2,21,22}. The efficient delivery of such therapeutic proteins to the CNS is a major focus in gene therapy. Genetically modified neuroepithelial cell lines integrate into the CNS more efficiently than fibroblasts, and these cells can express functional gene products *in vivo*^{38,39}. However, precursors to dopaminergic neurons, manipulated to express therapeutic levels of growth factors, could uniquely combine the potential of genetic manipulation and neuronal function in the grafted cells.

There is a considerable effort to characterize the molecular steps involved in the neuronal specification of CNS stem cells^{7,40,41}. We anticipate that the growing understanding of these mechanisms at a molecular level will lead to further improvements in the culture system reported here. However, CNS neurons derived from stem cells have not been analyzed on a functional level *in vivo*. The present study demonstrates that dopaminergic neurons can efficiently differentiate from expanded precursors and modify behavior after grafting. The expansion and differentiation steps lead to an estimated thirtyfold increase

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in the number of dopaminergic neurons over primary non-expanded control cultures and to an estimated sevenfold increase over the number of nigral TH-immunoreactive neurons present in the adult rat brain^{14,15}. In humans, the phase of cell birth is protracted (6 to 8.5 weeks after conception) compared to the period of dopaminergic neuronal differentiation in the rat (E12 to E15), and this might offer a longer period for cell expansion *in vitro*. The success in expanding dopaminergic precursors in rodents suggests that a similar approach should now be attempted with primate mesencephalic material and with precursors of other neuronal subpopulations. There was no indication of uncontrolled growth in any of the animals transplanted or in over two hundred culture series tested. However, larger cohorts of animals and long-term observations beyond three months after transplantation will be needed to conclusively assess the safety of expanded precursors in neural transplantation.

Methods

TISSUE CULTURE AND IMMUNOHISTOCHEMISTRY. Tissue was obtained from the ventral mesencephalon of E12 rat embryos (Sprague Dawley, plug day is day 0), as described⁴². Tissue pieces were centrifuged and mechanically triturated to quasi-single-cell suspension. 150–200 × 10³ cells per ml were plated at a concentration of 5 ml of cell suspension on a 10 cm dish precoated with polyornithine (15 µg per ml) and fibronectin (1 µg per ml). The medium, consisting of DMEM and F12 (Gibco) with N2 supplement, was changed every other day and bFGF (10 ng per ml) added daily. At the end of the expansion phase (6–8 days), dishes were incubated with HBSS, and cells were mechanically removed and resuspended to quasi-single-cell suspension. 200,000 cells per ml were distributed in 15 ml Falcon tubes (1 ml per tube) and placed in a roller drum (Bellco). This procedure typically resulted in the formation of one large (0.6–1.2 mm in diameter) sphere per tube after seven days of differentiation. The differentiation medium for reaggregate cultures consisted of Neurobasal[®] with 2% B27[®], with or without FBS (10%, Gibco), GDNF 10 ng per ml, BDNF 10 ng per ml, NT4/5 10 ng per ml (Peprotech) or SHH 2.5 µg per ml (kindly provided by Dr. Thomas Müller), depending on the individual experiments. BrdU (1 µM, Boehringer-Mannheim) was added to the medium at days 1, 3 and 5 of the expansion phase. Cells were marked after plating with a 3 mm circle (Nikon) on the bottom of the plate⁷ and followed by phase-contrast microscopy and daily photomicrographs. For TUNEL labeling, cells were fixed after 7 hours, 1 day, 2 days, 4 days or 8 days in 4% paraformaldehyde and 0.15% picric acid and labeled according to the specifications of the manufacturer (Boehringer-Mannheim). All negative controls (no deoxynucleotidyl transferase in the reaction mixture) were devoid of any labeling. Reggregates were fixed, equilibrated in 30% sucrose, cut into 20-µm sections on a freezing microtome (Microm HM500) and adhered to a gelatin-coated glass carrier. Standard immunohistochemical procedures were followed. The following antibodies were used: TH polyclonal 1:500 (Pel Freeze), dopamine polyclonal 1:500 (Chemicon), bFGF receptor monoclonal 1:100 (Sigma), β-tubulin type III (TuJ1) monoclonal 1:500 (Berkley Antibody Company), GABA polyclonal 1:2000 (SIGMA), serotonin polyclonal 1:8000 (SIGMA), ChAT polyclonal 1:500 (Chemicon), GFAP polyclonal 1:100 (Chemicon), galactocerebroside monoclonal 1:50 (Boehringer-Mannheim), TH monoclonal 1:10000 (Sigma), nestin #130 polyclonal 1:500 (M. Marvin and R.D.G.M.) and Ki-67 polyclonal 1:1000 (Novocastra). Dopamine staining was carried out on cultures fixed in 5% glutaraldehyde and 1% metabisulfite in Tris buffer and then kept in metabisulfite-containing solutions prior to incubation in the secondary antibody. BrdU immunohistochemistry was performed after postfixation in 95% ethanol and 5% glacial acetic acid and incubation in primary antibodies and nucleases (Amersham Life Science). Appropriate FITC- and LRSC-labeled secondary antibodies were used for double immunohistochemistry for TH and BrdU.

DOPAMINE DETERMINATION BY REVERSE-PHASE HPLC. The growth medium of expanded precursors from three independent culture series was collected after 5–7 days of differentiation under serum-free conditions and

immediately stabilized by adding 88 µl of 85% orthophosphoric acid and 4.4 mg of metabisulfite to each sample ml. Additional cultures of the same series were placed in HBSS for 25 minutes at 37°C either under basal conditions (HBSS only) or under stimulation with 56 mM KCl. The supernatant was collected and stabilized as above. Dopamine was extracted by aluminium adsorption as described⁴³. Separation of the injected samples (20 µl) was achieved by isocratic elution on a Hewlett-Packard Series 1050 HPLC system with a reverse-phase C18 column (3 µm particle size, 80 × 4.6 mm dimension, ESA, Inc.) in a commercially available MD-TM mobile phase (ESA Inc.). The flow rate was set at 1 ml per min, resulting in a working pressure of 100 bar and an elution time of 2.7 minutes for dopamine. The oxidative potential of the analytical cell (ESA Inc. Mod. 5011) was set at +325 mV. Results were validated by co-elution with dopamine standards under varying buffer conditions and detector settings. No dopamine was detected in unconditioned control medium and HBSS.

SURGICAL PROCEDURES AND BEHAVIORAL TESTING. Animals were housed and treated following NIH guidelines. Adult female Sprague-Dawley rats (200–250 g) were lesioned by unilateral injection of 6-hydroxydopamine bromide at two sites along the medial forebrain bundle⁴⁴. Six to seven reaggregates were loaded into a blunt 18G spinal needle (Sherwood Medical) and deposited at AP +1.0 mm, ML -2.5 mm and V -4.7 mm (coordinates relative to bregma), toothbar set at -2.5. Automated assessment (Rota Count-8, Columbus Instruments) of amphetamine-induced rotational behavior (i.p. injection of 5 mg d-amphetamine sulfate (SIGMA) per kg body weight) was carried out twice before transplantation (days -21 and -14) and three times after transplantation (days 30, 55 and 80). Only animals with stable pre-transplantation scores of over 7 rotations per minute (average 11 ± 2) were included in this study.

HISTOLOGICAL ASSESSMENT AND QUANTITATIVE ANALYSES. Total graft volume was estimated using Cavalieri's estimator⁴⁵. TH-immunoreactive cells were counted within the outlined graft area as used for volume estimation. Uniform randomly chosen sections of individual reaggregates were analyzed for the total number of TH-immunoreactive cells by means of a stereological grid (fractionator)⁴⁵. Conventional and confocal images were obtained with Axiophot and Axiovert microscopes, respectively (Zeiss). Confocal image stacks were acquired by single and dual wavelength excitation at 488 and 568 nm. Identically treated but not BrdU-incubated control cultures were used as a negative control for BrdU and TH double immunohistochemistry. Posttransplantation rotation scores were compared by the non-parametric Mann-Whitney U test. The effect of serum and growth factors was analyzed using ANOVA and Dunnett posthoc comparisons. Data are given as mean ± standard error.

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